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Catabolism of Collagen and Non-collagen Protein in the Rat Uterus during Post-partum Involution

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The mechanism by which proteins are catabolized has yet to be clearly defined. Certain lines of evidence indicate that the process is not a simple proteolysis, but that it requires energy and may involve a reversal of certain steps of protein synthesis (Walter, 1960).

The mammalian uterus is one of the most favourable tissues for the study of protein catabolism under normal physiological conditions because during the course of the involution which follows parturition, there is an exceedingly rapid breakdown of protein with little concomitant protein synthesis. Furthermore, a distinction can be made between the breakdown of two different types of protein: namely, extracellular, fibrous collagen which is relatively insoluble, and the non-collagenous proteins which are largely intracellular.

The breakdown of uterine collagen is of particular interest because collagen is generally considered to have a very slow rate of metabolic turnover (Neuberger & Slack, 1953). However, during uterine involution this protein may have a half-life as brief as 24 hr. (Harkness & Moralee, 1956). These authors have shown that there was no loss of collagen through the lumen of the uterus, pointing to a catabolism within the myometrium. A suggested mechanism of collagen breakdown *in vivo* has been provided by Jackson (1957*a*), who has presented evidence that collagen catabolism in the regressing carrageenin granuloma involves the preliminary conversion of fibrous into soluble forms of collagen.

The present investigation was designed to determine the steps that may be operative in protein degradation during uterine involution. Quantitative measurements of hydroxyproline were used as a means of identifying various possible products of collagen degradation, such as soluble collagens, small peptides and free amino acids in the uterus tissue as well as in blood and urine. Nitrogen, proline and glycine measurements were employed to follow the course of the rapid catabolism of the non-collagenous proteins of the uterus. A preliminary report of this research has been presented previously (Woessner, 1959).

EXPERIMENTAL

Tissue preparation. Pregnant female rats were obtained from the Sprague–Dawley Co. and maintained on stock diet in separate cages until appropriate times after parturition. Offspring were allowed to remain with the mother until the uteri were removed. It was usually possible to determine the time of delivery of the last offspring within 2 hr. The average rat body weight exclusive of uterine contents was 230 g. The rats were anaesthetized with ether and exsanguinated by heart puncture, the blood being saved for hydroxyproline measurements. The uterus and cervix were then dissected out rapidly and spread out in a Y-shape. A cut was made just below the junction of the two horns and the cervical portion was discarded. Small fatty nodules corresponding to each site of foetal attachment were cut off the surface of the uterus. Uteri in the earliest post-partum stages were slit open and rinsed to remove clotted blood; later uteri were not so treated. The uteri were blotted, weighed and fractionated as described below.

The uteri were homogenized in the VirTis macrohomogenizer (VirTis Co. Inc., Yonkers, N.Y.) in 0·9% NaCl and washed out into centrifuge tubes. The total volume of NaCl added was 10 ml. plus an additional 1 ml. for each 100 mg. wet wt. above 1 g. The tubes were stoppered and shaken horizontally for 18 hr. at 2°. The contents were centrifuged at 42 000*g* for 30 min. The pellet (fraction A) was hydrolysed in a sealed tube in 6N-HCl for 18 hr. at 110°. Portions (1 ml.) of the supernatant were hydrolysed directly by adding an equal volume of 12N-HCl (fraction B). Both hydrolysates (A and B) were assayed for nitrogen, proline, hydroxyproline and glycine. Further portions of the supernatant were dialysed against 5 vol. of distilled water. Portions of the diffusible fraction (fraction C) were assayed directly for free proline, hyproxyproline and glycine. Other portions of the diffusible fraction were dried on the steam table and hydrolysed in HCl as described above (fraction D). The hydrolysates were assayed for proline, hydroxyproline and glycine. Nitrogen determinations were performed by the Conway (1950) microdiffusion method applied to Kjeldahl digests. Glycine was determined by the method of Christensen, Riggs & Ray (1951) and proline by the method of Troll & Lindsley (1955). Hydroxyproline was assayed by a modified Stegemann method (Woessner, 1961, method II).

Calculations. Total nitrogen, hydroxyproline, proline and glycine were calculated from the sum of fractions A and B. Soluble collagen (i.e. soluble, non-diffusible forms of hydroxyproline) was estimated from the hydroxyproline values of fraction B minus D. The validity of this estima-

tion was checked in about 30% of the cases by independent measurement of exhaustively dialysed supernatants. A value for the small-peptide forms of the three amino acids was obtained from the difference: fraction D minus C.

Hydroxyproline values were converted into their collagen equivalents by multiplying by the factor 7·46 (Neuman & Logan, 1950). Collagen nitrogen was calculated by multiplying the amount of collagen (based on hydroxyproline) by 0·186 (Bowes, Elliott & Moss, 1955). The total non-collagen protein (fractions A and B) was then calculated by multiplying by 6·25 the total nitrogen values corrected for collagen nitrogen. The amounts of collagen proline and glycine were assumed to be 1·18 and 2·0 times the hydroxyproline values (Bowes *et al.* 1955). Non-collagen proline and glycine values were calculated by correcting total values for the collagen contribution.

The experimental results have been presented graphically in two forms: as the amount per entire uterus and as the concentration (per g. wet wt. of tissue). Not all of the parameters change at the same rate as wet weight. This gives rise to changes in concentration. However, concentration data alone do not distinguish between concentration increases due to an increase in the amount of a substance and those due to a slower loss of that substance compared with the loss of wet weight. The data showing amounts per uterus resolve the problem in some cases by revealing increases in absolute amounts. This form of the data also reveals the magnitude of the involutionary changes.

Blood. Samples were allowed to clot and portions of the resulting serum were dialysed against 5 vol. of distilled water. The diffusible fractions were analysed for hydroxyproline before and after acid hydrolysis. The sac contents were exhaustively dialysed, hydrolysed and assayed for hydroxyproline (Woessner, 1961).

Urine. To examine hydroxyproline excretion in the urine, 24 hr. samples of urine were collected from four pregnant rats. The collections were continued until 7 days after parturition. Offspring were removed from the cage at birth. The total urine output/24 hr. was acidified with HCl to 6N and hydrolysed overnight in the autoclave at 15 lb./in.^{2} pressure. The hydrolysates were evaporated to dryness and reconstituted in water. Hydroxyproline was determined by the method of Wiss (1949). Unhydrolysed urine was also tested for the presence of free hydroxyproline.

Proteolytic activity. Uterine homogenates were tested for their catheptic activity against haemoglobin, uterine proteins and uterine collagen. Haemoglobin digestion was studied by the method of Anson (1938). The pH of the acidified haemoglobin solution was adjusted to various values with NaOH and then tris–acetate buffers were added. The mixtures were incubated for 30 min. at 37°. The digestion of uterine proteins was tested in a similar fashion by adjusting homogenates to various pH values, adding appropriate tris–acetate buffers and allowing autolysis to proceed for 30 min. at 37°. For collagen-digestion studies, homogenates were distributed in centrifuge tubes and uniform pellets were obtained by centrifuging for 30 min. at 42 000*g*. Buffers were added to each pellet and portions of the supernatant, adjusted to appropriate pH values, were added. The tubes were stoppered and shaken in a horizontal position for 18 hr. at 37°. Next the tubes were centrifuged at 42000*g* for 30 min. and the supernatant was assayed for hydroxyproline after acid hydrolysis. Pellets incubated with buffer in place of supernatant or at 2° instead of 37° served as blanks for solubilization effects as distinct from digestion. Prolidase and prolinase were assayed and the results expressed as previously described (Woessner & Boucek, 1959).

RESULTS

Disappearance of collagen from the uterus. The changes in total hydroxyproline content of the uterus during involution are shown in Fig. 1 (*A*). These hydroxyproline values represent all forms of hydroxyproline present in the uterus, including small peptides, free hydroxyproline and elastin. For all practical purposes, however, the hydroxyproline values may be equated directly with the collagen content of the uterus, since the free, small-peptide and elastin forms of hydroxyproline never exceed 5% of the total at any stage of involution.

Fig. 1(*A*) shows that there was a slight lag in hydroxyproline loss during the first 12–20 hr. *post partum*, followed by a rapid loss which was complete by 100 hr. If the hydroxyproline values are converted into their collagen equivalents by multiplying by 7·46, the results are found to be very closely comparable with those of Harkness & Moralee (1956), both in absolute values and in the

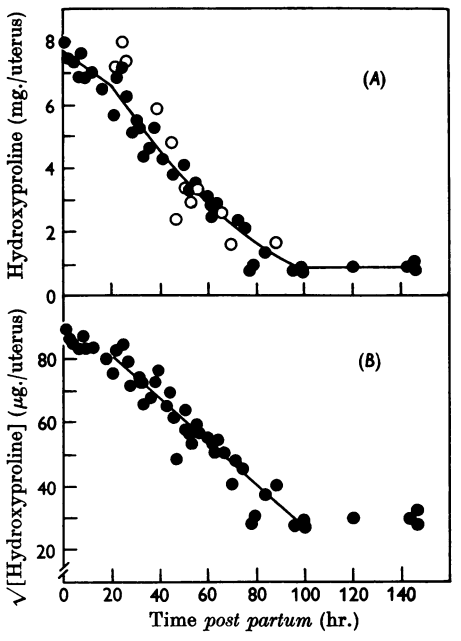

Fig. 1. (*A*) Total hydroxyproline of the rat uterus during *post partum* involution. (*B*) Square root of the total hydroxyproline. ●, Uteri containing 10–12 foetuses; ○, uteri containing more than 12 foetuses.

rate of decrease. An effect of the number of foetuses per uterus on the collagen content during the first 2 days of involution was also observed in the present work (cf. Harkness & Moralee, 1956); uteri with more than 12 foetuses (Fig. 1*A*) had appreciably higher collagen contents than uteri with 10–12 foetuses. For this reason the three points at 22, 25 and 26 hr. (14, 13 and 15 foetuses respectively) were not included when the curves in Fig. 1 (*A*) and (*B*) were drawn. With the exception of these three points there is close uniformity of the data, which is attributed to the selection of only those uteri containing 10 or more foetuses.

Harkness & Moralee consider the loss of collagen to be a first-order process between 24 and 96 hr., but I could not fit a first-order curve to the results from this period. However, a fairly close linear relationship was found between the square root of the total hydroxyproline content and the time *post partum* (Fig. 1*B*). This is the type of relationship that would be anticipated if collagen is being removed from the surface of fibres (see Discussion). The least-squares straight line from Fig. 1 (*B*) ($Y = 94{\cdot}33 - 0{\cdot}6699X$) was used to calculate the curve fitted to the region 20–100 hr. in Fig. 1 (*A*).

To test whether uterine-collagen breakdown involved the conversion of fibrous collagen into soluble forms (as suggested by the findings of Jackson, 1957*a*, *b*, in carrageenin granuloma), a soluble-collagen fraction was prepared by extraction with 0·9 % sodium chloride soln. The hydroxyproline content of this fraction is shown in Fig. 2 (*A*). It was found that there was an increase in the total amount of soluble collagen per uterus, a peak being reached by 40–50 hr. *post partum*. The concentration of soluble collagen (Fig. 2*B*) was much more markedly elevated, reaching a peak at 60 hr. This concentration increase is the resultant of two factors: the increase in amount of soluble collagen and the rapid loss of wet weight (Fig. 6*A*). However, this soluble-collagen fraction never exceeded 1 % of the total collagen content of the uterus at any stage of involution.

To test the completeness of extraction afforded by the single saline extraction always employed, several uteri, removed at different intervals after delivery, were subjected to two additional saline (0·45 M) extractions, followed by three extractions with 0·5 M-acetic acid. Only trace amounts of hydroxyproline were obtained by further saline extraction, but an additional 1·5 % of the total hydroxyproline was recovered in the combined acetic acid extracts.

If collagen is being broken down by hydrolytic processes in the uterus, one might expect the occurrence of peptide fragments. These could be readily identified by their hydroxyproline content. It was found, however, that hydroxyproline in the form of diffusible peptides amounted to less than 5 μg./uterus and showed no significant change during the course of involution.

The most striking changes observed during involution were in free hydroxyproline content. There was a doubling of the amount per uterus (Fig. 3*A*) between 0 and 48 hr. *post partum*. On a wet-weight basis (Fig. 3*B*) it is seen that the concentration of free hydroxyproline increased by a factor of six from term to 60–70 hr. *post partum*. In the period between 60 and 80 hr. free hydroxyproline constituted 2·5 % of the total hydroxyproline of the uterus. By the fifth day the free hydroxyproline returned to the value found at parturition. This was maintained to 15 days *post partum* and was also the value found in nulliparous uteri (see below).

Fate of free hydroxyproline. This marked increase in free hydroxyproline suggests that the uterine collagen may be catabolized, at least in part, to the free amino acid stage. The question of the final disposition of the hydroxyproline then arises.

An examination of the serum hydroxyproline was undertaken to see if hydroxyproline was leaving the uterus through the circulation. Less than 5 % of the serum hydroxyproline was in the form of small peptides. Non-diffusible hydroxyproline could not be detected by the assay and therefore must have been less than 2 μg./ml. Free

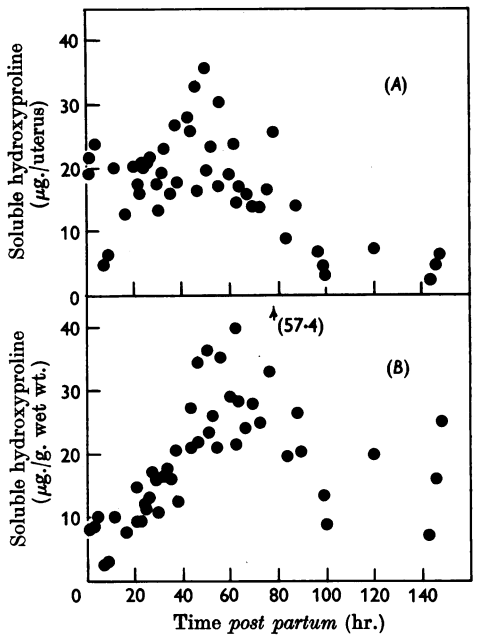

Fig. 2. (*A*) Soluble, non-diffusible hydroxyproline content of the involuting rat uterus. (*B*) Concentration of soluble, non-diffusible hydroxyproline.

hydroxyproline was the major form found in serum. It did not show a marked peak corresponding to that found in the uterus, but there was a significant elevation in this fraction during the period of involution. Rats (41) in the period 0–96 hr. *post partum* had $8{\cdot}02 \pm 2{\cdot}18\,\mu$g. of hydroxyproline/ml. of serum, compared with a value of $6{\cdot}50 \pm 1{\cdot}37$ for the serum of 25 rats in the period beyond 96 hr. This difference is significant at $P < 0{\cdot}01$.

Urinary hydroxyproline occurred almost entirely in bound forms. Only 3–4 % of the urinary hydroxyproline could be detected before acid hydrolysis (i.e. free hydroxyproline); the remainder was in the form of diffusible peptides. The results from hydrolysed urine show that only a small portion of the collagen hydroxyproline was excreted as urinary hydroxyproline. If all the hydroxyproline disappearing from the uterus had appeared in the urine, the urinary excretion would have had to rise to a total of 2·5 mg./day. The mean value for the 5 days preceding parturition is $610 \pm 240\,\mu$g. of hydroxyproline/day, compared with 790 ± 240 during the 5 days after the day of parturition. The post-partum urinary-peptide hydroxyproline is thus elevated by about 30 %, but the difference is significant only at $P < 0{\cdot}05$.

Non-collagen protein. Wet weight and non-collagen protein changed in a pattern markedly distinct from that of collagen. For wet weight (Fig. 4*A*) the involution process started immediately after delivery and there was no detectable lag period. Non-collagen nitrogen, non-collagen proline and non-collagen glycine, free proline and free glycine, all showed the same pattern as wet weight (Figs. 4*B*, 5*A*, 5*B*, 6*A*, 7*A*). Changes in each of these parameters could be described by first-order-rate equations over 0–100 hr. Figs. 4–7 show the theoretical curves obtained by plotting the data as log parameter against time and calculating the least-squares best-fit straight line. The results of these calculations are summarized in Table 1. The wet weight, non-collagen nitrogen and non-collagen proline and non-collagen glycine all decreased, with a half-life of about 36 hr. Free glycine and proline, including that derived from collagen, decreased at a somewhat lower rate.

The curves for wet weight and non-collagen nitrogen, non-collagen proline and non-collagen glycine do not exhibit the gradual approach to asymptotic values normally associated with first-order reactions. Rather, beyond 100 hr. the points depart markedly from the straight line of the logarithmic plots and fall slightly above the extension of the calculated curves in Figs. 4 and 5. It appears that there is an abrupt change in these involution processes beyond 100 hr. This change is not seen, however, in the free proline and glycine (Figs. 6*A* and 7*A*), which continued to decrease at a constant rate to 150 hr. *post partum*.

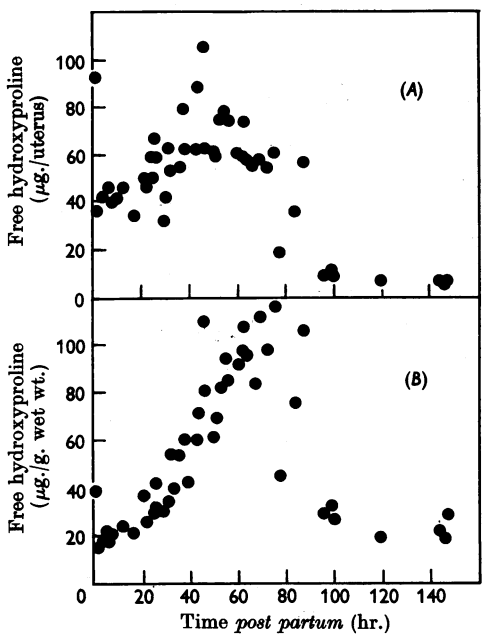

Fig. 3. Amount (*A*) and concentration (*B*) of free hydroxyproline in the uterus.

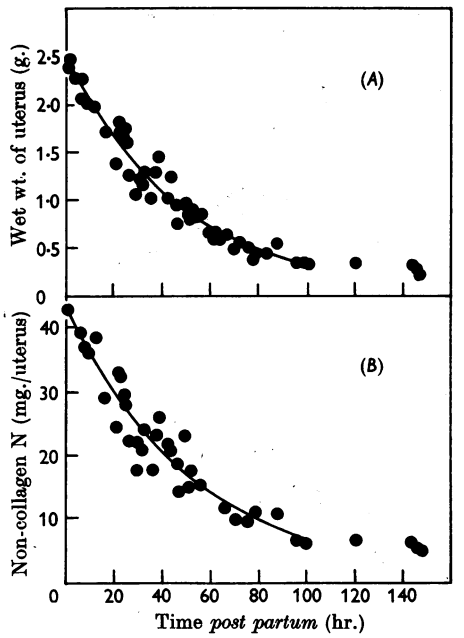

Fig. 4. (*A*) Change in wet weight of uterus during involution. (*B*) Change in non-collagen nitrogen during involution.

Since the non-collagen nitrogen, non-collagen proline and non-collagen glycine decreased at the same rate as wet weight, they exhibited no concentration change during involution. Free proline and glycine, however, decreased more slowly than the wet weight, resulting in an increased concentration of these free amino acids (Figs. 6*B* and 7*B*), which persisted for at least 15 days *post partum* (Table 2).

Small-peptide forms of proline (not illustrated) behaved very similarly to peptide hydroxyproline, maintaining a constant value of 40 μg./uterus with a wide scattering of values. Small-peptide glycine was quite similar to free glycine. It dropped from 200 μg./uterus at term to about 45 μg. at 150 hr. and showed the same increase in concentration as did free glycine (three- to four-fold).

Magnitude of involutionary changes. Table 2 shows the values of the various parameters for nulliparous uteri. If these are compared with the corresponding values from the initial portion of each curve (0–10 hr. *post partum*; Figs. 1–7), the magnitude of the changes occurring during the course of pregnancy can be determined. Table 2 also shows the average values for the period 6–14 days *post partum*. These values may be compared with the terminal portions of the curves in Figs. 1–7 as a measure of further involutionary change and with results from the nulliparous uteri as a measure of the return to initial conditions.

Pregnancy results in an increase of the total collagen content by a factor of 3·5 (Fig. 1*A* and Table 2). This is in close agreement with the results of Needham & Cawkwell (1957). The involutionary resorption of collagen is completed in 4 days. This,

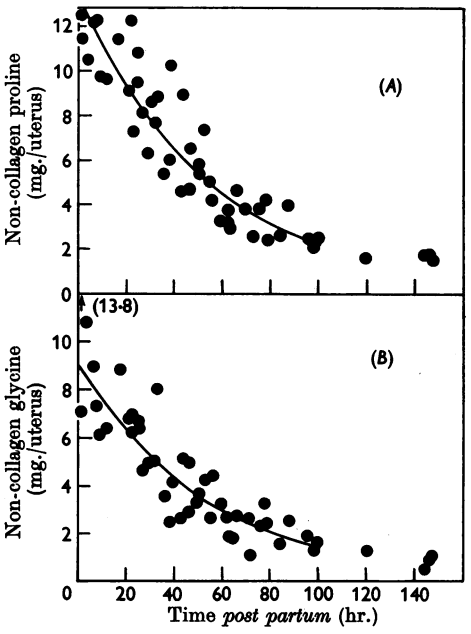

Fig. 5. Non-collagen-proline (*A*) and non-collagen-glycine (*B*) disappearance from involuting uterus.

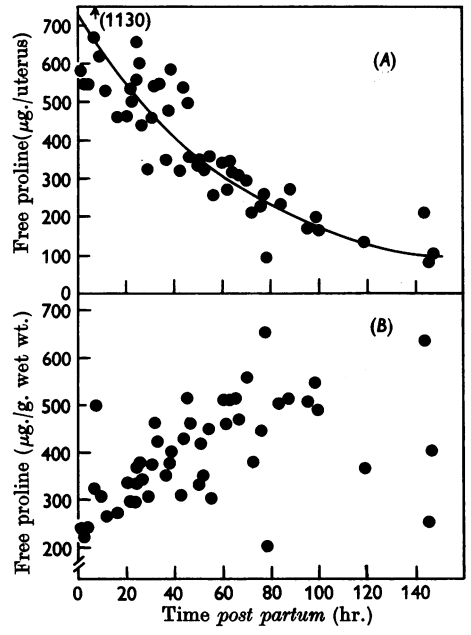

Fig. 6. Amount (*A*) and concentration (*B*) of free proline in involuting uterus.

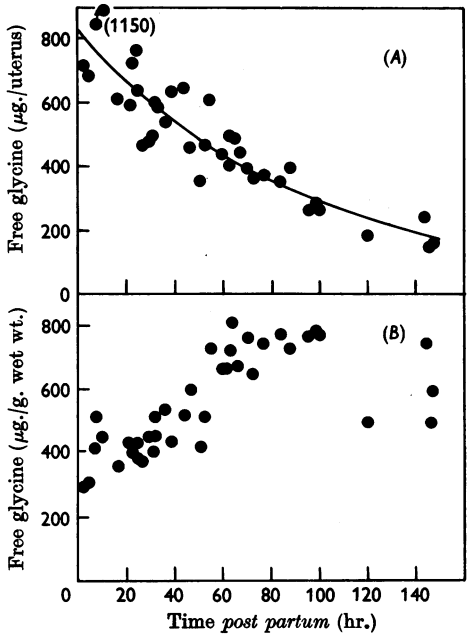

Fig. 7. Amount (*A*) and concentration (*B*) of free glycine in involuting uterus.

Table 1. *Results of the first-order rate plots of the logarithms of parameters versus time* post partum

Parameter (wt./uterus)	Coefficient of correlation	K* (ln/hr.)	Half-life (hr.)
Wet weight	−0·98	0·0205	33·8
Non-collagen nitrogen	−0·96	0·0184	37·6
Non-collagen proline	−0·92	0·0179	38·6
Non-collagen glycine	−0·88	0·0187	37·1
Free proline	−0·85	0·0142	48·8
Free glycine	−0·88	0·0106	65·6

* First-order rate constant in terms of natural logarithms and hours.

Table 2. *Summary of results for the period of involution between* 150 *and* 350 *hr.* post partum *and for nulliparous controls*

Numbers of animals used are given in parentheses. Five animals were used for the nulliparous group.

	At 150–350 hr. *post partum*	Nulliparous group
Total hydroxyproline (μg./uterus)	1130 (11)	2270
Soluble, non-diffusible hydroxyproline (μg./uterus)	5·4 (8)	12·0
Soluble, non-diffusible hydroxyproline (μg./g. wet wt.)	19·3 (8)	27·7
Diffusible peptide hydroxyproline (μg./uterus)	3·3 (8)	0·8
Diffusible peptide hydroxyproline (μg./g. wet wt.)	12·6 (8)	1·7
Free hydroxyproline (μg./uterus)	4·9 (7)	9·4
Free hydroxyproline (μg./g. wet wt.)	20·0 (7)	21·8
Wet wt. (g.)	0·30 (12)	0·46
Non-collagen nitrogen (mg./uterus)	5·9 (10)	—
Non-collagen proline (μg./uterus)	1600 (7)	1130
Non-collagen glycine (μg./uterus)	1390 (7)	1700
Free proline (μg./uterus)	159 (7)	102
Free glycine (μg./uterus)	195 (7)	—

however, is not the end of the change in collagen, since at 100 hr. *post partum* only one-half of the normal (nulliparous) amount of collagen remains. There is some new formation of collagen, but this has not progressed very far by 15 days *post partum*. The overshooting of the nulliparous base line during involution is also found in several of the other parameters (Table 2).

The amount of soluble collagen (non-diffusible hydroxyproline) per uterus also falls to about one-half of the nulliparous value (Table 2), but the change is not so marked when considered on the basis of concentration. Small-peptide hydroxyproline remains elevated to 15 days, but this represents an extremely small fraction of the total hydroxyproline. Free hydroxyproline differs from the other forms of hydroxyproline in that it returns to the same concentration as in the nulliparous uterus within 5 days *post partum*.

Proteolytic activities. To determine whether proteolytic enzymes might be responsible for the rapid breakdown of collagen and other proteins, uterine homogenates were assayed for proteolytic activity. Protein breakdown in incubated homogenates of involuting uterus showed two pH optima: a major activity at pH 3·5 and a smaller activity at pH 8·5 (Fig. 8). In contrast, digestion of haemoglobin by homogenate supernatants (15 min., 2000g) occurred only at acid pH values.

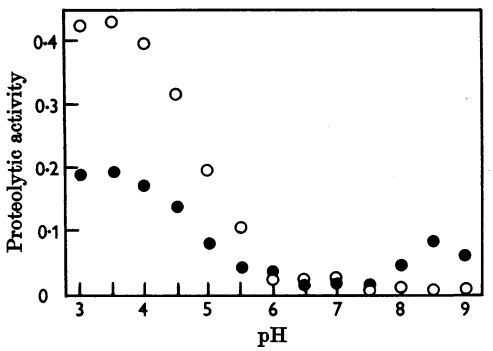

Fig. 8. Proteolytic activity of homogenates of involuting uterus at 37°, expressed in arbitrary units. ○, Digestion of denatured haemoglobin in a system containing 0·5 ml. of homogenate and 2·0 ml. of buffered haemoglobin; ●, autodigestion of uterine protein (0·5 ml. of homogenate plus 1 ml. of buffer).

Autolytic digestion of uterine collagen was measured in homogenates of four uteri removed 48 hr. *post partum*. An average digestion of 94% was obtained after incubation for 18 hr. at pH 3·5 and 37°. That is, 94% of the collagen was converted

into soluble forms that could not be sedimented by centrifuging at 42 000*g* for 30 min. Controls incubated at 37° with buffer in place of uterine supernatant or at 2° with supernatant showed only 3–4 % solubilization. That true proteolytic digestion to peptides had occurred was further supported by the finding that 53 % of the solubilized hydroxyproline was diffusible.

The uterine homogenates also were found to contain dipeptidase activity of the type involved in the breakdown of peptide sequences typical of collagen. Prolidase (imidodipeptidase) and prolinase (iminodipeptidase) activities were determined in uteri 28 hr. *post partum*. The prolidase activity was 1·05 units/g. wet wt. of tissue. A unit is defined as the first-order rate constant K (in terms of decimal logarithms and minutes), X the final dilution of enzyme in the assay system X ml. of enzyme/g. of tissue. The prolinase activity was 62 μmoles of substrate/hr./g. wet wt. Both enzymes are present at about one-third of the activity found in rat liver. The prolidase activity is similar to that found in sponge-implant connective tissue (Woessner & Boucek, 1959), whereas the prolinase activity is 20 times as high. By approximating the first-order reaction of the prolidase activity to a zero-order reaction, a comparison of the two enzymes may be made. Prolidase is about five times as active as prolinase. This is the same ratio of activities as that found in human-uterus muscle by Smith (1948).

DISCUSSION

Rate of collagen catabolism. The rate of collagen removal between 20 and 100 hr. *post partum* was found to be proportional to the square root of the total collagen concentration (Fig. 1*B*). A mechanism of removal may be postulated which accounts for this relationship.

Let C_0 = amount of collagen at zero time (t_0); C = amount of collagen at t; x = amount of collagen digested at t. At any time t, $C = C_0 - x$.

Assume that the rate of digestion is proportional to the surface area of the collagen fibres:

$$dx/dt = K(2\pi rl),$$

where r = fibre radius, l = fibre length, K = rate constant.

Assuming that the collagen fibres have relatively uniform diameters and that the fibre ends make a negligible contribution to the total area, one may treat the total collagen as a single fibre of unit length.

Thus $$C = \pi r^2 l.$$

Solving for r in terms of C:

$$r = \sqrt{(C/\pi l)}.$$

Substituting this in the rate expression and combining constants:

$$dx/dt = K'\sqrt{C}.$$

Integration between the limits $t = 0$ and $t = t$ yields:

$$\sqrt{C_0} - \sqrt{(C_0 - x)} = K''t.$$

A plot of $\sqrt{(C_0 - x)}$ against t should yield a straight line with slope $= -K''$ and y intercept $= \sqrt{C_0}$.

Fig. 1 (*B*) is such a plot, and the close fit of the experimental points to the straight line between 20 and 100 hr. supports the interpretation that the digestion rate is governed by the surface area of the collagen fibres. The surface which governs the rate may be that of the fibre bundles, of the individual fibres or of the submicroscopic fibrils. The uniformity of fibre diameters postulated in the derivation of the rate equation would be most likely to be encountered at the fibrillar level (cf. Jackson, 1956).

Inspection of Fig. 1 (*A*) shows that a linear relation between amount of collagen and time might also be considered. However, this interpretation is not favoured, for several reasons. Such a straight line does not fit the data in the region 18–100 hr. as closely as does the square-root function. Moreover, it obscures the initial lag period of about 18 hr., a phenomenon shown more clearly by the data of Harkness & Moralee (1956). It is also unusual to find a process following zero-order kinetics during the consumption of 85 % of the substrate and continuing to the point of cessation of the reaction.

The postulate that the rate of collagen removal is proportional to the surface area of the fibres receives added support from histological studies of other types of collagen removal. In regression of liver cirrhosis (Morrione, 1949) and carrageenin granuloma (Williams, 1957) collagen fibres grow progressively thinner as involution proceeds.

During pregnancy there is a progressive thickening of the collagen fibres of the uterus. The individual fibres appear swollen, hypertrophied and much more argyrophilic (Maibenco, 1960; Bassett, 1958). If pregnancy involves a thickening, and involution a thinning, of the fibres, then the abrupt cessation of involution beyond 100 hr. may be due to the cessation of digestion when a resistant inner core of the fibres is reached. This resistant core would correspond to the inner portion of the fibres present before pregnancy; the outer portion of the pre-existing fibres would have to be removed to account for the overshoot beyond the nulliparous base line (Table 2) in involution. The possibility of a resistant core is supported by the finding that post-partum involution and the slow involution caused by ovariectomy both lead to the same final value for collagen per uterus (Harkness, Harkness & Moralee, 1956).

On the other hand, certain lines of evidence suggest that the newly formed collagen fibres are enzymically indistinguishable from the pre-existing collagen. In this case, the cessation of involution beyond 100 hr. would depend not on the consumption of available substrate, physically or chemically different from the residual collagen, but on some physiological change in the animal. This idea is supported by the abrupt changes in loss of wet weight and non-collagen protein beyond 100 hr., by the finding that the rate of collagen loss is a function of the total collagen content, by the failure to find any unusual solubility properties of the newly formed collagen and by the almost complete digestion of collagen obtained *in vitro* at pH 3·5. Hormonal changes may provide the limiting physiological mechanism. Dawson (1946) has shown that parturition is followed by a sudden drop in oestrogen concentrations. Lactation prolongs the depression of oestrogen, leading to hyperinvolution. In the absence of lactation, oestrogen rises earlier and involution does not proceed to the full extent. Ovariectomy leads to collagen removal from the rat uterus (Harkness *et al.* 1956) and oestrogen administration leads to collagen formation in the rat uterus (Harkness, Harkness & Moralee, 1957). A rising concentration of oestrogen might be invoked to explain the cessation of involution beyond 100 hr. *post partum*.

Intermediates in collagen catabolism. The present study supports the hypothesis of Jackson (1957*a*) that the first step in collagen resorption is the conversion of fibrous collagen into soluble collagen. It is important to note that in spite of the small amounts of soluble collagen in the involuting uterus this fraction increases in amount per uterus during involution. This is in contrast with the finding in the granuloma that there was a large increase in the proportion of collagen in the soluble forms, but not an increase in the absolute amount of soluble collagen per granuloma (Jackson, 1957*b*). Thus a possible explanation of the increased proportion of soluble collagens could be that these were removed more slowly than the fibrous forms of collagen. Although this appears unlikely, at least the uterine changes are not open to this interpretation.

An explanation for the low concentrations of soluble collagen in the uterus may be that the solubilization step is the rate-limiting step in catabolism of uterine collagen with the result that there is little opportunity for an extensive accumulation of soluble collagens. This may be the reason for the rate of collagen catabolism appearing to be a function of fibre area: only those molecules at the surface would be in a position to be solubilized. This concept may explain the failure to find increased concentrations of soluble collagen during the rapid disappearance of collagen from sponge-implant tissue in guinea pigs after removal of ascorbic acid from the diet (Gould, Manner, Goldman & Stolman, 1960). A slightly more rapid removal of solubilized collagen would prevent even such a small increase in soluble collagen as was found in the uterus. In the carrageenin granuloma, on the other hand, removal of soluble collagen would appear to be the rate-limiting step.

Although the evidence points to a final production of free hydroxyproline from collagen, it could not be determined if this step involves small-peptide intermediates. The hydroxyproline-containing peptides form an almost negligible portion of the total hydroxyproline and no significant change in the amount of peptide/uterus could be found. The demonstrated peptidase activities in the uterus may be sufficient to prevent an accumulation of hydroxyproline-containing peptide intermediates. Studies of free hydroxyproline indicate that collagen degradation is carried completely to the free amino acid stage. The total amount of free hydroxyproline doubles and the concentration increases by a factor of 6. The free hydroxyproline comprises as much as 2·5% of the total uterine hydroxyproline. This is an unusually high amount of free hydroxyproline compared with other tissues of the rat (Woessner, 1961).

The concept that collagen is degraded to the free amino acid stage receives further support from the finding of an elevation of serum hydroxyproline, the failure to find appreciable amounts of hydroxyproline peptides in serum and the lack of any marked rise in urinary-peptide hydroxyproline. Although it might be possible that many proteins are taken apart by a reversal of the synthetic steps yielding activated amino acids or re-utilizable peptide fragments (Walter, 1960) this would not be expected with collagen since hydroxyproline (and probably peptides containing hydroxyproline) cannot be utilized in protein synthesis (Stetten, 1949).

The free hydroxyproline does not accumulate beyond a certain amount, pointing either to further catabolism in the uterus or to removal by the blood stream. The increased serum concentration of hydroxyproline points to the latter hypothesis as being more likely. The failure to find elevated urinary free hydroxyproline is easily explained by the known metabolism of injected or fed hydroxyproline (e.g. Wolf & Berger, 1958).

More interesting is the relatively small increase in urinary-peptide hydroxyproline, when viewed in comparison with the large amount of collagen being degraded. Urinary hydroxyproline is believed to provide a direct reflexion of collagen metabolism (Prockop & Sjoerdsma, 1961). The present findings indicate that less than 15% of the products of

collagen breakdown appear in the urine as hydroxyproline peptides, if indeed, the observed increase is considered significant ($P < 0.05$). The uterine hydroxyproline peptides, only 5 μg. of hydroxyproline/uterus, do not increase in amount in involution. There is little detectable small-peptide hydroxyproline in the blood (approx. 0·2 μg./ml.). Thus it is not clear that the increased urinary-peptide hydroxyproline is related to the breakdown of uterine collagen; it might be derived from other collagen pools in the body under the influence of the marked hormonal changes occurring immediately after parturition.

Non-collagen protein catabolism. The non-collagen proteins have a pattern of catabolism distinct from that of collagen. The total non-collagen nitrogen, non-collagen proline and non-collagen glycine disappear from the uterus at a rate proportional to their concentration, whereas the rate of collagen removal was proportional to the square root of its concentration. Furthermore, there was an initial lag period in the removal of collagen, suggesting a period of mobilization or activation of certain cell types involved in collagen breakdown. The non-collagen proteins were attacked immediately with no discernible lag. Since uterine-muscle growth in pregnancy occurs almost entirely by hypertrophy of the cells with little change in cell numbers (Maibenco, 1960), whereas involution involves a shrinkage or volume reduction of muscle cells (Kuramitsu & Loeb, 1921; Dawson, 1946), it is likely that the rapid first-order reactions observed in our study characterize the process of the muscle cells divesting themselves of the extra cytoplasm added during the hypertrophic growth of pregnancy. Note that the rate is first-order with respect to the total substrate. No simple rate expression is obtained if one considers only that portion of the substrate added during pregnancy. This indicates that the involution processes probably do not distinguish between pre-existing proteins and proteins newly formed in pregnancy.

The first-order rate kinetics cease to apply beyond 100 hr. Since it is unlikely that the available substrate is completely consumed by this time, there would appear to be a relatively abrupt change in the organism leading to the cessation of involution. This change may be hormonally controlled (see above).

The evidence for small-peptide intermediates in the breakdown of non-collagen protein is equivocal. Although the small-peptide forms of proline and glycine increased in concentration during involution, they did not increase in amount above that found at parturition. The increase in concentration might therefore be attributed to some mechanism resulting in a slower loss of peptides than of protein and wet weight.

It is possible that free amino acids are the end product of catabolism of non-collagen protein. The concentration increase in free proline and glycine occasioned by the slower rate of loss compared with the other components of the uterus (Table 2) and the continuation of this loss beyond 100 hr. when the other involutionary processes have ceased, support the hypothesis that free amino acids are the end product of catabolism. On the other hand, the rate of decrease of free glycine and proline may be slowed by the contribution of these amino acids coming from collagen breakdown. This, together with the failure to observe any increase in the absolute amounts of free amino acids, leaves the question unresolved.

Role of proteases in collagen breakdown. The rat uterus contains at least two proteases, having pH optima at pH 3·5 and 8·5. The acid cathepsin is much more active than the alkaline, particularly when haemoglobin is the substrate. At pH 3·5 and 37° there is almost complete digestion of uterine collagen by homogenates. This digestion is not merely a 'solubilization' effect produced by the breakage of a few bonds; rather the collagen is digested to the peptide stage. A similar enzyme activity from human uterus has already been characterized (Woessner & Brewer, 1960).

It is not necessary to postulate that the uterine protease is a specific collagenase. It may act, first of all, to increase the solubilization of the fibres either by attacking a few bonds of the collagen or by removing part of the matrix surrounding the collagen (cf. Grant & Alburn, 1960; Jackson, 1953). Once the collagen has been solubilized it may undergo denaturation at 37° (cf. Kazakova, Orekhovich & Shpikiter, 1958). Such a denatured 'procollagen' would be susceptible to further attack by relatively non-specific proteinases. Here again the rate of solubilization may play a key role in determining the overall kinetics of collagen catabolism.

Might the acid cathepsin found in uterine homogenates be involved in the catabolism of collagen and other proteins *in vivo*? Acid cathepsins are known to be elevated in a number of types of tissue regression, including involution of the rat uterus and mammary gland (Greenbaum & Greenwood, 1954) and the Flexner–Jobling carcinoma (Fodor, Funk & Tomashefsky, 1955). It has been shown that acid cathepsins are frequently localized in subcellular particles termed lysosomes, and that these lysosomes may empty their contents into digestive vacuoles within the cytoplasm (de Duve, 1959). Preliminary experiments in this Laboratory show that at least part of the rat-uterus cathepsin is lysosomal. This enzyme might function by acting on ingested collagen fragments or, more likely, on regions of collagen fibrils which have been com-

pletely surrounded by the cell. For example, Wessel (1959) has demonstrated that decidual cells attack collagen by wrapping themselves around the fibrils so that the collagen appears to be intracellular. The attainment of the requisite acid pH values might be feasible in confined regions at the surface of the fibre (McLaren & Babcock, 1959).

Uterine collagen may possess special properties that render it susceptible to proteolysis or other mechanisms of metabolic breakdown. The collagen of non-pregnant rat uterus had the highest metabolic turnover of a number of tissues investigated (Kao, Hilker & McGavack, 1961). Human uterine collagen has a lower thermal-shrinkage temperature than other human collagens and retains its fluorescence until the menopause (Brown, Consden & Glynn, 1958). Moreover, the new collagen deposited during pregnancy has altered tinctorial properties (Bassett, 1958) and would be expected to be loosely bonded. It was, in fact, surprising that no unusual solubility properties were found in the present study.

The final proposed step in collagen degradation, the digestion of peptides to amino acids, poses no problems since the uterus contains peptidases able to digest the unusual peptides, such as prolylhydroxyproline and hydroxyprolylglycine, that might be expected to arise from collagen. The presence of these peptidases at relatively high activities may also account for the failure to find any accumulation of small hydroxyproline-containing peptides during involution.

The various lines of evidence discussed above suggest the following tentative scheme of collagen breakdown in uterine involution:

(1) Collagen fibres undergo solubilization at a rate proportional to fibre-surface area. This rate-limiting process is probably enzymic and dependent on cell types such as the histiocytes.

(2) Solubilized collagen undergoes thermal denaturation at 37°, rendering it susceptible to proteolytic enzymes.

(3) Acid cathepsins, probably lysosomal in origin, complete the breakdown of collagen to peptides.

(4) The peptides or at least hydroxyproline-containing peptides, are degraded by peptidases to free amino acids.

This scheme is presented as a basis for further investigation of the many unresolved aspects of collagen degradation.

SUMMARY

1. The metabolic breakdown of collagen and non-collagen protein was studied in the rat uterus undergoing post-partum involution.

2. Collagen disappeared from the uterus at a rate proportional to the surface area of the fibre, after an initial lag period of about 18 hr. The process was complete by 100 hr. *post partum*.

3. A small, but significant, increase in the amount of saline-soluble collagen occurred, reaching a peak at 40–50 hr. *post partum*.

4. Small peptides containing hydroxyproline did not increase in amount during involution.

5. Free hydroxyproline doubled in amount and increased sixfold in concentration, reaching a peak about 60 hr. *post partum*. This free hydroxyproline appears to leave the uterus through the blood stream.

6. Only a small increase in urinary-peptide hydroxyproline could be detected.

7. Non-collagen protein and wet weight decreased at first-order rates with half-lives of 34–38 hr. Free proline and glycine decreased at significantly slower rates (49 and 66 hr.).

8. The involuting uterus contains two catheptic activities. The cathepsin having optimum pH 3·5 can effect almost complete digestion of uterine collagen at 37° *in vitro*. Imino- and imido-dipeptidase activities were one-third of those found in rat liver.

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REFERENCES

Anson, M. L. (1938). *J. gen. Physiol.* **22**, 79.
Bassett, E. G. (1958). *Nature, Lond.*, **181**, 196.
Bowes, J. H., Elliott, R. G. & Moss, J. A. (1955). *Biochem. J.* **61**, 143.
Brown, P. C., Consden, R. & Glynn, L. E. (1958). *Ann. Rheum. Dis.* **17**, 196.
Christensen, H. N., Riggs, T. R. & Ray, N. E. (1951). *Analyt. Chem.* **23**, 1521.
Conway, E. J. (1950). *Microdiffusion Analysis and Volumetric Error*, p. 124. London: Crosby Lockwood and Son, Ltd.
Dawson, A. B. (1946). *Amer. J. Anat.* **79**, 241.
de Duve, C. (1959). *Subcellular Particles*, p. 128. New York: Ronald Press.
Fodor, P. J., Funk, C. & Tomashefsky, P. (1955). *Arch. Biochem. Biophys.* **56**, 281.
Gould, B. S., Manner, G., Goldman, H. M. & Stolman, J. (1960). *Ann. N.Y. Acad. Sci.* **85**, 385.
Grant, N. H. & Alburn, H. E. (1960). *Arch. Biochem. Biophys.* **89**, 262.
Greenbaum, A. L. & Greenwood, F. C. (1954). *Biochem. J.* **56**, 625.
Harkness, M. L. R., Harkness, R. D. & Moralee, B. E. (1956). *Quart. J. exp. Physiol.* **41**, 254.
Harkness, M. L. R., Harkness, R. D. & Moralee, B. E. (1957). *J. Physiol.* **135**, 270.

Harkness, R. D. & Moralee, B. E. (1956). *J. Physiol.* **132**, 502.
Jackson, D. S. (1953). *Biochem. J.* **54**, 638.
Jackson, D. S. (1957*a*). *Connective Tissue*, p. 62. Oxford: Blackwell Scientific Publications Ltd.
Jackson, D. S. (1957*b*). *Biochem. J.* **65**, 277.
Jackson, S. F. (1956). *Proc. Roy. Soc.* B, **144**, 556.
Kao, K.-Y. T., Hilker, D. M. & McGavack, T. H. (1961). *Proc. Soc. exp. Biol., N.Y.*, **106**, 121.
Kazakova, O. V., Orekhovich, V. N. & Shpikiter, V. O. (1958). *Dokl. Akad. Nauk, SSSR*, **120**, 359.
Kuramitsu, C. & Loeb, L. (1921). *Amer. J. Physiol.* **4**, 422.
McLaren, A. D. & Babcock, K. L. (1959). *Subcellular Particles*, p. 23. New York: Ronald Press.
Maibenco, H. G. (1960). *Anat. Rec.* **136**, 59.
Morrione, T. G. (1949). *Amer. J. Path.* **25**, 273.
Needham, D. M. & Cawkwell, J. M. (1957). *Biochem. J.* **65**, 540.
Neuberger, A. & Slack, H. G. B. (1953). *Biochem. J.* **53**, 47.
Neuman, R. E. & Logan, M. A. (1950). *J. biol. Chem.* **186**, 549.
Prockop, D. J. & Sjoerdsma, A. (1961). *J. clin. Invest.* **40**, 843.
Smith, E. L. (1948). *J. biol. Chem.* **173**, 553.
Stetten, M. R. (1949). *J. biol. Chem.* **181**, 31.
Troll, W. & Lindsley, J. (1955). *J. biol. Chem.* **215**, 655.
Walter, H. (1960). *Nature, Lond.*, **188**, 643.
Wessel, W. (1959). *Virchows Arch.* **332**, 224.
Williams. G. (1957). *J. Path. Bact.* **73**, 557.
Wiss, O. (1949). *Helv. chim. acta*, **32**, 149.
Woessner, J. F., jun. (1959). *Fed. Proc.* **18**, 461.
Woessner, J. F., jun. (1961). *Arch. Biochem. Biophys.* **93**, 440.
Woessner, J. F., jun. & Boucek, R. J. (1959). *J. biol. Chem.* **234**, 3296.
Woessner, J. F., jun. & Brewer, T. H. (1960). *Fed. Proc.* **19**, 335.
Wolf, G. & Berger, C. R. A. (1958). *J. biol. Chem.* **230**, 231.

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The Relationship between Glutathione and Protein Sulphydryl Groups in Germinating Pea Seeds

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After the work of Hopkins & Morgan (1943), Spragg & Yemm (1959) showed that in the early stages of germination of pea seeds there was a rapid and quantitative conversion of GSSG into GSH, and it was suggested that the production of large quantities of GSH may precede the reduction of protein disulphide groups.

The activities of many enzymes depend on the presence of free chemical groups on the protein chains. Some enzymes, e.g. the glycolytic enzymes, depend on the sulphydryl group, oxidation of which to the disulphide form inactivates the enzymes [Hopkins, Morgan & Lutwak-Mann (1938), with GSSG as oxidizing agent]. In contrast, White (1960) showed that the activity of ribonuclease was a function of the number of disulphide bonds in the protein chain, and Liener (1957) found a similar relationship for the activity of trypsin.

Results from experiments *in vitro* show that reactions can occur between protein disulphide groups and GSH; for instance, Narahara &Williams (1959) reduced the disulphide bonds of insulin with GSH in the presence of an enzyme prepared from liver. In addition to enzymically induced reactions, Ryle & Sanger (1955) showed that an interchange occurred between disulphide groups in the presence of acid, and Huggins, Topley & Jensen (1951) suggested that a sulphydryl–disulphide interaction took place when albumins were dissolved in urea solution. This evidence suggests that the redox state of the protein sulphydryl groups can be readily affected by other thiols.

It appears possible that in a cell the balance between the activities of different classes of enzymes could be controlled, in part, by the oxidation–reduction state of the sulphydryl group alone, and the present study was made to obtain information on the reaction between glutathione and the protein sulphydryl or disulphide groups in the intact cell.

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MATERIALS AND METHODS

Plant materials. Pea seeds (var. Meteor) were used. They were surface-sterilized with 3% (w/v) calcium hypochlorite, and were washed with sterile water before germination was started. This was taken as the start of an experiment and all times were measured from this point. The seeds were germinated in an aerated solution or in